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CAUTION: Always use filter tips and bleach everything!!!

Large Scale Prep

Reagents:

DMEM-2% FBS, L-glut, Pen/Strep

DMEM-10% FBS, L-glut, Pen/Strep

T75s

Dry ice/MeOH bath

Protocol:

I. Plating Cells

Plate 293 cells in T75s so that at time of infection, cells are 50-60% confluent.

10 The number of flasks you choose to infect depends upon (1) how much virus you have to do the infection and (2) the yield you want.

11 To keep the number of defective particles as low as possible, it is critical that original stocks (or as close as you can get) are used to generate new viral stocks. Eventually, the virus will need to be re-plaque purified.

12 I had very little of the original viral stocks so I infected with an MOI of 1. Higher titer viruses apparently can be achieved by infecting with higher MOIs (5-10).

13 As a guideline, infection of 2-3 T75s at an MOI of 1 has yielded 2-3 ml of 10^9 PFU/ml.

14 293 cells seem to have a lag, so that if you plate 2.5 X 10^6 cells sometime between noon and 5 pm, and count the next day, you will find you have exactly the same number of cells. Therefore, plate the number of cells that you want to have the next day. In the past, I was aiming for 50% confluent flasks to give the cells room to grow before they were killed (plating 5 X 10^6 cells/T75 yielded <50% confluency next day; I would try plating 6-7 X 10^6 cells/T75). However, the two times I prepared virus, all the cells lifted off the flask before confluency could be reached, so I don't know if this matters.

II. Infect

Calculate amount of virus to add. For example, if plan to infect with an MOI of 1, and plated 6 X 10^6 cells, add 6 X 10^6 PFU. (I guess at cell number, no need to be exact for this).

Thaw virus on ice.

Add virus to DMEM-2. Use 4 ml per T75 (using a low volume is critical to getting good infection).

Wash cells with PBS.

Add virus mix to cells.
Incubate at 37°C for 3 hours. Rock flask occasionally.
Add 10 ml DMEM-10 to cells.
Harvest virus 3 days post-infection. The original protocol based upon Vogelstein says to harvest the virus when 1/3 to 1/2 of the cells are detached, usually 3-5 days post-infection. However, by 3 days post-infection, 100% of my cells have detached. It may be dependent upon the subclone of 293 cells I have. I think the best thing to do is to achieve a balance between spread of infection and cell death, so I do the minimum – which is 3 days.

III. Harvest

The virus is cell associated, so need to harvest the cells, which will most likely all be detached, and then release the virus by freeze-thawing the cells.
Harvest the cells by transferring culture medium (if need to, bang the flask to release cells) to 50 ml conical tubes.
Pellet cells and resuspend pellet in sterile PBS. Use 1 ml/T75.
Freeze cells in dry ice/MEOH bath.
Thaw at 37°C, as soon as thawed put on ice.
Vortex vigorously.
Repeat freeze/thaw/vortex 3X for a total of 4X.
Spin samples and collect supernatent.
Aliquot supernatent and store at –80°C.

Quick MOI Test

Reagents:

DMEM-2% FBS, L-glut, Pen/Strep

DMEM-10% FBS, L-glut, Pen/Strep

6-well plates

Protocol:

Plate 293 cells in 6 wells. Need 1 plate/virus being titered, plus one extra well to trypsinize and count the day of infection. Want approximately 1 X 10^6 cells/well at time of infection (so will probably need to plate 1 X 10^6 cells/well). Wells will look <50% confluent.

Remove culture medium from plate. Add 500 µl DMEM-2%.

Add 0, 2, 5, 10, 25, 50 µl virus supt to the wells. When I tested stocks that turned out to be 10^9 PFU/ml, the lowest volume gave complete CPE. So, you may need to adjust the volumes to give more confidence to the results (see below). However, my line of 293 cells ball up very easily, so that may be the problem.

Incubate plates for 3 hrs at 37°C. Rock occasionally.

Add 1.5 ml DMEM-10 to each well.

Incubate for 72 hrs.

Check for CPE.

The lowest volume of virus added to the well which produces total CPE 3 days pi corresponds to an MOI of 10-20. Thus, knowing the number of cells that were infected, you can estimate the amount of virus present in the supernatent.

$TCID_{50}$ Titration

Reagents:

DMEM-2% FBS, L-glut, Pen/Strep

96-well flat bottom plates.

Small snap-cap tubes.

Protocol (for titering 1 virus stock):

Trypsinize 293 cells and count. Prepare 20 ml of 10^5 cells/ml in DMEM-2. Dispense 100 µl (10^4 cells) per well in 2 96-well plates. Put in incubator.
Add DMEM-2 to snap-cap tubes for serial dilutions. Prepare *in duplicate*..
10^{-1} 0.9 ml
10^{-2} to 10^{-12} 1.8 ml
Thaw virus supt on ice.
Add 100 µl virus supt to the 10^{-1} tube.
Pipet up and down 5 times to mix.
Change tip *(absolutely critical!!)*.
Take 200 µl out of 10^{-1} and add to 10^{-2}. Repeat all the way to 10^{-12}.
Add 100 µl per well of 10^{-5} to 10^{-12} as in picture (10 wells/dilution). If add highest dilution (10^{-12}) to lowest dilution (10^{-5}), can use the same tip.
Add 100 µl DMEM-2 to control wells.
Incubate at 37°C for 10 days.
Score wells for CPE. Count wells even if only have a small spot of CPE. For the experiment to be valid, *all the control wells must show no CPE. Also, the lowest dilution must show CPE in all wells, and the highest dilution must show no CPE in any wells*.
Calculate titer as in example.

Note: when I titered viruses that were 10^9 PFU/ml, I found that the appropriate range of dilutions to plate was 10^{-5} to 10^{-12}. Adjust as needed.

Example

											control	
	1	2	3	4	5	6	7	8	9	10	11	12
10^{-12}												
10^{-11}												
10^{-10}												
10^{-9}												
10^{-8}			CPE				CPE					
10^{-7}	CPE		CPE		CPE	CPE		CPE		CPE		
10^{-6}	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE		
10^{-5}	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE	CPE		

Results

Determine the ratio of positive wells per row. You must include the lower dilutions that were not plated in the calculations.

Dilution	Ratio
10^{-12}	0/10=0
10^{-11}	0/10=0
10^{-10}	0/10=0
10^{-9}	0/10=0
10^{-8}	2/10=0.2
10^{-7}	6/10=0.6
10^{-6}	10/10=1
10^{-5}	10/10=1
10^{-4}	10/10=1
10^{-3}	10/10=1
10^{-2}	10/10=1
10^{-1}	10/10=1

For 100 µl of dilution, the titer it $T = 10^{1 + d(S-0.5)}$

D = Log 10 of the dilution (=1 for a ten-fold dilution)
S = the sum of ratios (always starting from the first 10^{-1} dilution) =
1+1+1+1+1+1+0.6+0.2=6.8

$T = 10^{1+(6.8-0.5)} = 10^{7.3}$ (*for 100 µl of virus*)
$T = 10^{8.3}$ $TCID_{50}$/ml

The titer as measured by $TCID_{50}$ is 0.7 Log higher than the titer by standard plaque assay. To transform $TCID_{50}$/ml into PFU/ml:

$T = 1 \times 10^{8.3}$ $TCID_{50}$/ml
$= 1 \times 10^{8.3-0.7}$ PFU/ml

$= 1 \text{ X } 10^{7.6}$ PFU/ml $= 4 \text{ x } 10^{7}$ PFU/ml [use the 10^{X} key on calculator to convert]
Between duplicates, the difference in titers should be <0.7 Log.

Infection of MNT-1 Cells

This experiment is designed specifically for infection of MNT-1 cells with adeno-PDX and adeno-α1 AT. 3 dishes are infected with each virus: 2 for biochemistry, and 1 for EM.

Reagents:

Trypsin

DMEM-2% FBS, L-glut, Pen/Strep

MNT-1 medium (DMEM-20% FBS, Pen/Strep, L-glut, NaPyruvate, 10% AIM-V, NEAA)

10 cm dishes (done in dishes instead of flasks so that can scrape fixed cells for EM)

Protocol:

Trypsinize MNT-1 cells and count.
Plate 3 X 10^6 cells/10 cm dish. Plate 7 dishes (3 per virus plus 1 extra to count) [This resulted in confluent cells at time of harvest – may want to plate less.] Add coverslips to check infection efficiency by IFM.
Next day, trypsinize and count 1 dish to determine cell number.
Using the cell number, calculate the amount of virus you will add. To get expression of the α1 AT and the PDX, you must co-infect with adeno-trans at a 1:1 ratio.

- *For the next experiment, we want to infect with an MOI of 20 for the α1 AT and an MOI of 5 for the PDX to compensate for the defective particles (use virus prep#1 prepared 3-1-01). Not sure what to do about the trans. We could add more trans to keep the same total MOI, but I think keeping it at a 1:1 ratio would ensure that we don't get massive overexpression of PDX.*

Thaw virus on ice.
Add virus to 3 ml DMEM-2/dish.
Wash cells with PBS
Add virus-DMEM mix to cells.
Incubate 3 hrs at 37°C with occasional rocking.
Add 7 ml MNT-1 medium to each dish.
Incubate at 37°C for 48 hrs.

Adenovirus Stocks

Original stocks from Gary Thomas
liquid N2 tower E rack 3

- adeno trans 3.45×10^7
- adeno α1 AT assumed to be 1×10^8
- adeno PDX 5.56×10^7

Prep 1 3-1-01
–80 rack D box 17

- adeno trans 4.08×10^9
- adeno α1 AT 4×10^9
- adeno PDX 2.25×10^9

Prep 2 3-26-01
–80 rack D box 17

- adeno trans 8.95×10^9
- adeno α1 AT 1.19×10^9
- adeno PDX 1.42×10^9